Effect of ErbB4 on Triple-Negative Breast Cancer Cell Growth & Migration

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Abstract

Members of the ErbB subfamily of receptor tyrosine kinases are critical regulators of normal mammary gland development, and alterations in their signaling have been associated with breast tumorigenesis. ErbB4 expression in breast carcinomas predicts improved patient survival, and minimal expression has been noted in the triple-negative breast cancer (TNBC) subtype, which is associated with poor prognosis. The goal of this study was to examine the effects of ErbB4 overexpression on the growth and migration of TNBC cell lines. A green fluorescent protein (GFP)-containing construct was used to overexpress ErbB4 in the ErbB4-negative TNBC cell lines BT-20, BT-549 and MDA-MB-468. An empty vector construct was used as the control. Expression was confirmed by western blot and fluorescence microscopy to detect expression of ErbB4 or GFP respectively. Cell motility and growth was assessed with a transwell migration assay and a sulforhodamine B assay, respectively. Our data indicates that overexpression of ErbB4 resulted in no significant difference in the migration of BT-549 or MDA-MB-468 cells but resulted in a slight increase in the migration of BT-20 cells. ErbB4 had a growth inhibitory effect on BT-549 and BT-20 cells but showed no difference in the growth of MDA-MB-468 cells. This data suggests multiple ErbB4-mediated mechanisms occur to alter TNBC cell growth.

Introduction

Breast cancer is the most common malignancy among women in the United States. Over 200,000 women were newly diagnosed with invasive breast cancer and 40,000 patients died from their disease in 2010. Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer in which the tumor cells lack expression of estrogen, progesterone, and HER2 (ErbB2) receptors. Patients with TNBC have limited treatment options and, on average, lower 5-year survival rates. The ErbB/HER/EGFR family has long been implicated in various human cancers, principally due to its role in regulating cell survival, proliferation, motility, and growth (Figure 1).

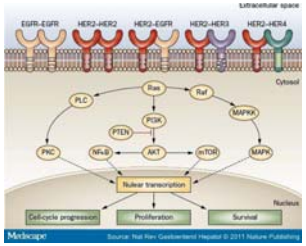

Figure 1: ErbB4 (HER4)is a receptor tyrosine kinase and thus follows the typical pattern of receptor dimerization and autophosphorylation upon ligand binding.

ErbB4 is the last member in this family of receptors to be discovered, and recent studies indicate that it may serve a function contrasting with that of its related receptors, ErbB1, 2 and -3. Despite the known oncogenic potential of the ErbB family, mounting evidence points to a possible tumor-suppressor-like role for ErbB4. This finding has been confounded, however, with conflicting reports regarding the function of ErbB4 within the past decade.

Introduction (cont.)

One of the primary functions of ErbB4 in vivo is in the terminal differentiation of mammary glands during late-pregnancy and lactation induction. Parity and prolonged lactation are known factors that reduce breast cancer risk. ErbB4 expression has been associated with cell growth inhibition, a relatively well differentiated histopathological grade, in addition to estrogen and progesterone receptor positivity. All of these findings indicate a more favorable prognosis, supporting the protein's tumor suppressive role. However, overexpression of ErbB4 was shown to transform rodent mammary epithelial cells and induce tumorigenesis. A precise explanation of ErbB4 activities and mechanisms of action remains elusive to this day.

Methods

Plasmid transfection

pCMV6-AC-GFP plasmid, containing the ERBB4 gene, was obtained from Origene Technologies. The plasmid was transformed in Top10 competent bacterial cells. The next day, several bacteria colonies were selected for insert screening. Large scale plasmid purifications were carried out using the Qiagen maxiprep kit. Empty vectors are used as controls. The TNBC cell lines used were BT-20, BT-549, and MDA-MB-468, all of which lack expression of ErbB4. All cell lines were cultured in media containing 10% fetal bovine serum. The cells were seeded the day prior to transfection to produce a starting cell confluency of approximately 60 percent. On the day of transfection, cells were replaced with fresh media and the X-tremeGENE HP DNA Transfection Reagent protocol was followed. Following incubation of at least 18 h, these cells were either lysed to run a western blot or counted to proceed with the growth and migration assays.

Growth assay

The sulforhodamine B (SRB) assay was used to assess cell density. The growth plates were seeded at a concentration of 1000 cells/well (5 cells/µl) and were left to incubate at 37°C. Six replicates of both the ErbB4-transfected and the empty vector-transfected cells were prepared for each cell line. Growth progression was measured at days 1, 3, 5, and 7 post-seeding. The cells were fixed on those days and eventually stained and solubilized before measurements were taken. A solution of 50% trichloroacetic acid (TCA) was used to fix the wells. The plates were then allowed to incubate for 1 h at 4°C before they were rinsed thoroughly in water and allowed to dry. After ensuring that the plates have completely dried, the SRB cell staining assay was performed using 0.04% SRB as the reagent. The plates were left at room temperature for 20 min before being thoroughly rinsed in a 1% acetic acid wash and allowed to dry. Lastly, the cells were solubilized in 10 mM Tris solution. The wells were briefly agitated to ensure complete homogeneity, and the samples were analyzed with a spectrophotometer at an absorbance of 565 nm. From this data a graph was generated, plotting the growth of each cell line over the week. This process was repeated to produce three trials.

Migration assay

The CytoSelectTM 96-well cell migration assay kit was used to assess cell motility. The sterile migration plate consists of three parts: a plate cover, membrane chamber, and a feeder tray on the bottom. 50,000 cells were seeded per well (500 cells/µl), and a sterile 0.5% bovine serum albumin (BSA) solution was also added to the cell suspension. No chemoattractant was used, rather each cell line's respective media was added to the wells of the feeder tray.

Methods (cont.)

Migration assay (cont.)

Four replicates of each condition (ErbB4 or empty vector) were prepared for each cell line. The membrane chamber was then placed atop the feeder tray and the desired concentration of BSA and cell solution was added before allowing the plate to incubate at 37°C overnight. The following day, the cell detachment solution was added to the cell harvesting tray. After discarding the residual fluid in the membrane chamber, this chamber was placed into the cell detachment solution. Following 30 min of incubation at 37°C, a lysis buffer/dye solution was added to the mixture and the plate was read with a fluorescence plate reader at 480 nm/520 nm. The data generated by this machine similarly allows a graph to be plotted measuring migration of the cell lines, each compared to an empty control.

Results: Experiment 1

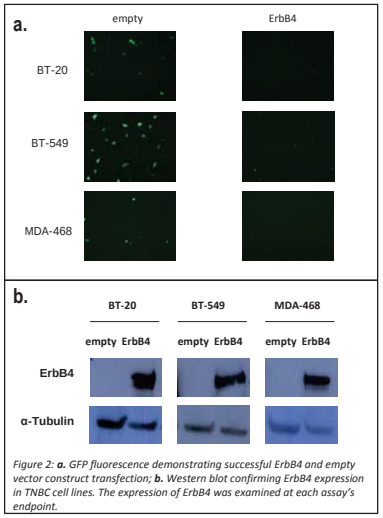

*Figure 2: **a.** GFP fluorescence demonstrating successful ErbB4 and empty vector construct transfection; **b.** Western blot confirming ErbB4 expression in TNBC cell lines. The expression of ErbB4 was examined at each assay's endpoint.*

Results: Experiment 2

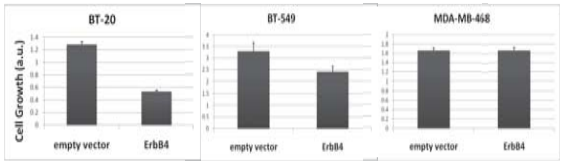

Figure 3: Graph of TNBC cell growth using SRB assay. These results depict normalized data for cell growth on day 5 post-seeding across the 3 TNBC cell lines tested. In the BT-20 and BT-549 cell lines, ErbB4 exhibited a growth inhibitory character, while in the MDA-MB-468 cell line, ErbB4 had no significant effect on cell growth.

Results: Experiment 3

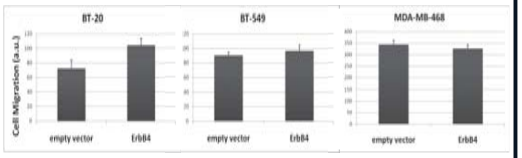

Figure 4: Graph of TNBC cell migration. These results depict cell migration approximately 24 h after seeding the cells in a transwell migration assay. ErbB4 enhanced migration in the BT-20 cell line, while resulting in no significant change in cell motility in the BT-549 and MDA-MB-468 cell lines.

Discussion and Conclusions

- The loss of ErbB4 is a frequent event in TNBC which occurs through various genomic mechanisms.
- The TNBC cell lines were transfected with either ERBB4 or empty vector expression constructs. GFP fluorescent microscopy as well as western blot confirmed the expression of ErbB4 in TNBC cell lines.
- Our study demonstrated that overexpression of ErbB4 enhanced migration of BT-20 cells, but yielded no significant difference in the migration of BT-549 or MDA-MB-468 cells compared to the empty vector control.
- Furthermore, ErbB4 over-expression inhibited growth of BT-20 and BT-549 cells, while causing no significant change in growth of MDA-MB-468 cells.
- Our study adds to the scarce amount literature on the role of ErbB4 and triple-negative breast cancer and sets a foundation for additional functional and mechanistic studies.
- Weaknesses of the study include both limitations of the assays used, e.g. the SRB growth assay lacks sensitivity to distinguish cell death from cell proliferation, as well as limitations in scope, e.g. subcellular localization of ErbB4 and the influences of the other ErbB receptors were not investigated during our experiments.
- The next steps include performing additional functional and mechanistic studies to further elucidate the role of ErbB4 in TNBC. These studies are intended to lead to clinically meaningful findings.

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